Effects of Systemic versus Local Administration of Corticosteroids on Mucosal Tolerance


*J Immunol* 2012; 188:470-476; Prepublished online 21 November 2011; doi: 10.4049/jimmunol.1101405

http://www.jimmunol.org/content/188/1/470

---

Why *The JI*?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**  This article *cites 38 articles*, 18 of which you can access for free at:  
http://www.jimmunol.org/content/188/1/470.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Effects of Systemic versus Local Administration of Corticosteroids on Mucosal Tolerance

Jerome Kerzerho,*1 Daniela Wunsch,†,1 Natacha Szely,* Hellmuth-Alexander Meyer,† Lisa Lurz,† Lars Röse,‡ Ulrich Wahn,† Omid Akbari,* and Philippe Stock‡

Respiratory exposure to allergen induces T cell tolerance and protection against the development of airway hyperactivity in animal models of asthma. Whereas systemic administration of dexamethasone during the delivery of respiratory Ag has been suggested to prevent the development of mucosal tolerance, the effects of local administration of corticosteroids, first-line treatment for patients with bronchial asthma, on mucosal tolerance remain unknown. To analyze the effects of systemic versus local administration of different types of corticosteroids on the development of mucosal tolerance, mice were exposed to respiratory allergen to induce mucosal tolerance with or without systemic or intranasal application of different doses of dexamethasone or prednisolone. After the induction of mucosal tolerance, proliferation of T cells was inhibited in tolerized mice, whereas systemic applications of corticosteroids restored T cell proliferation and secretion of Th2 cytokines. In contrast, inhaled corticosteroids showed no effect on both T cell proliferation and cytokine secretion. In addition, mice systemically treated with corticosteroids showed an increased airway hyperactivity with a significant lung inflammation, but also an increased T effector cells/regulatory T cells ratio in the second lymphoid organs when compared with mice that receive corticosteroids by inhalation. These results demonstrate that local administration of corticosteroids has no effect on the development of immune tolerance in contrast to systemically applied corticosteroids. Furthermore, although different concentrations of corticosteroids are administered to patients, our results demonstrated that the route of administration rather than the doses affects the effect of corticosteroids on respiratory tolerance induction. Considering the broad application of corticosteroids in patients with allergic disease and asthma, the route of administration of steroid substances seems crucial in terms of treatment and potential side effects. These findings may help elucidate the apparently contradicting results of corticosteroid treatment in allergic diseases. The Journal of Immunology, 2012, 188: 470–476.
**Materials and Methods**

**Mice**

Female BALB/cByj and DO11.10 BALB/c mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free mouse colony at the Keck School of Medicine, University of Southern California, and University Hospital Charité of Berlin under protocols approved by the Institutional Animal Care and Use Committee.

**Induction of mucosal tolerance and administration of CS**

Mucosal tolerance was induced by i.n. instillation of 100 μg OVA (Worthington Biochemical, Lakewood, NJ; chromatographically purified) in 50 μl PBS on consecutive 3 d (day 1, day 2, day 3). A group of mice was also treated i.p. with dexamethasone (Dexa.; 25 or 100 μg), prednisolone (Pred.; 500 or 125 μg; ICN, Aurora, OH) once per day on days 0 and 2, or i.n. with Dexa. (25 or 100 μg) and Pred. (125 or 500 μg; ICN) once per day on the days of induction of tolerance. Control mice received i.n. PBS. Subsequently, mice were immunized 9 d later i.p. with 50 μg OVA with 2 mg alum [Al(OH)₃], in a volume of 200 μl. On day 22, mice were sacrificed, and spleen, lung, and lymph nodes (LN) were collected.

**In vitro proliferation assays**

Peribronchial LN or spleen cells were harvested and passed through a nylon mesh, and stimulated in round-bottom 96-well plates (5 × 10⁶ cells/well) with or without increasing doses of OVA (as indicated in the figures) in 0.2 ml/well RPMI 1640 (Sigma-Aldrich), which contained FCS (10%), l-glutamine (2 mM), 2-ME (50 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). For measurement of cell proliferation, the cultures were pulsed with 0.25 μCi [³²P]thymidine for 16 h, and the incorporated radioactivity was measured in a Betaplate scintillation counter (MicroBeta Trilux; Wallac, Turku, Finland).

**Induction of AHR and measurement of airway responsiveness**

For measurement of AHR, mice were immunized, as described previously, and challenged with 100 μg LPS-free OVA Ag (Worthington Biochemical) in alumin administered i.p. After 8 d, mice were exposed to OVA (50 μg, i.n.) for 3 consecutive days. AHR responses were assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics, Troy, NY), as described before (11). In some experiments, we assessed AHR by invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated using a modified version of a described method (12). Aerosolized methacholine was administered in increasing concentrations of methacholine, and lung resistance (Rₐ) and dynamic compliance (Cdyn) by fitting flow, volume, and pressure to an equation of motion. AHR was measured at 24 h after terminal i.n. challenge.

**Collection of bronchoalveolar lavage fluid and lung histology**

After the measurement of AHR, the trachea was cannulated, the lung was lavaged twice with 1 ml PBS plus 2% FCS, and the fluids were pooled, as previously described (8). The relative number of leukocyte populations was differentiated on slide preparations of bronchoalveolar lavage (BAL) fluid stained with H&E. After the BAL, performed, lungs were removed, washed with PBS, fixed in 10% formalin, and stained with periodic acid Schiff (PAS) and H&E.

**Flow cytometry analysis**

Spleens, lungs, and draining LN were collected and digested with collagenase and DNase I, as previously described (8). Cells were preincubated with rat serum and washed before staining. For the identification of the T effector (CD4⁺CD25⁺Foxp3⁺) and Treg (CD4⁺CD25⁺Foxp3⁻) CD4⁺ DO11.10 T cells, 5 × 10⁶ cells were stained with an Ab combination that included allophycocyanin 7-Cy7–conjugated CD4 (clone L3T4; BD Biosciences), according to the protocol. Analytical flow cytometry was carried out using a FACS Canto II eight-color flow cytometry (BD Biosciences) on ice using standard procedures. Intracellular staining of Foxp3 was performed using the Foxp3 allophycocyanin staining kit (eBioscience), according to the manufacturer’s instructions. Serum levels of total and OVA-specific IgE were measured by ELISA, as previously described (13). Levels of OVA-specific IgE were related to pooled standards generated in our laboratories and expressed as arbitrary units per milliliter.

**Results**

**Systemic administration of CS blocks i.n. tolerance**

We previously demonstrated that respiratory exposure to allergen induces T cell tolerance, high levels of total and allergen-specific Cytokines were analyzed in cell culture supernatants using the Ready Set Go kit (mouse IL-4, IL-10, and IFN-γ; eBioscience), according to the manufacturer’s instructions. Serum levels of total and OVA-specific IgE were measured by ELISA, as previously described (13). Levels of OVA-specific IgE were related to pooled standards generated in our laboratories and expressed as arbitrary units per milliliter.

**Effect of CS to mucosal tolerance.** A group of BALB/c mice was sensitized, as described in A. Briefly, a group of BALB/c mice (n = 4) was treated i.n. with 100 μg OVA (OVA Tolerance) or PBS (OVA Sensitized) on consecutive 3 d (days 1–3). Some of the mice from the OVA tolerance group were also treated i.p. with 500 or 125 μg Pred. (OVA + Pred. 500 or 125) or 25 or 100 μg Dexa. (OVA + Dexa. 100 or 25) each on days 1 and 1 or i.n. with 500 or 125 μg Pred. (OVA + Pred. 500 or 125) or 25 or 100 μg Dexa. (OVA + Dexa. 25 or 100) once per day on the days of induction of tolerance. Subsequently, on day 12, all the mice were immunized in i.p. with OVA (50 μg) in alum (2 mg). On day 22, blood was withdrawn and serum levels of OVA-specific IgE were determined by ELISA (B). Data are mean ± SD of triplicate cultures, four mice per group (*p < 0.001). Subsequently, mice were sacrificed and splenocytes were restimulated in vitro with indicated doses of OVA for 48 h, and then pulsed with [³¹H]thymidine. Figure represents the thymidine incorporation after 8 h of incubation for the splenocytes from mice treated i.p. (C) or i.n. (D) with CS. Data are mean ± SD of triplicate cultures, three mice per group (*p < 0.01).
IgE, and protection against the development of AHR (9, 14). We also previously showed that systemic administration of Dexa. may enhance Th2 cytokine synthesis by reducing IL-12 production in APCs, which may lead to increased Th2 responses of CD4+ T cells (10). We therefore examined the effects of i.p. administration of CS on the development of i.n. tolerance. We induced OVA tolerance in groups of mice in presence or absence of systemic administration of different doses of Dexa. or Pred. during tolerance induction, as described in Fig. 1A. As expected, pretreatment of mice by i.n. exposure to OVA reduced the observed level of total and OVA-specific IgE to ~25% (Fig. 1B) and inhibited the subsequent proliferation of OVA-specific CD4+ T cells upon in vitro restimulation with OVA (Fig. 1C) compared with OVA-sensitized animals without mucosal tolerance induction. However, systemic administration of Dexa. or Pred. during the induction of respiratory tolerance increased serum OVA-specific IgE levels (Fig. 1B) and reversed the respiratory tolerance, as seen by the restoration of the in vitro proliferative response of OVA-specific T cells (Fig. 1C). In addition, we found that the capacity of i.p. CS to break the respiratory tolerance is dose dependent: we observed the in vitro proliferative response of OVA-specific T cells to be restored less efficiently with low systemic doses of Dexa. or Pred. (25 and 125 µg) than with high doses (100 and 500 µg) (Fig 1C). In addition, whereas pretreatment by i.n. exposure to OVA inhibited the development of AHR, mice systemically treated with Dexa. or Pred. during the induction phase of tolerance developed severe AHR measured as Penh (Fig. 2A) or as Rl and Cdyn in anesthetized, tracheostomized, and mechanically ventilated mice (Fig. 2B). Moreover, examination of the lungs and the BAL of mice i.p. treated with CS also showed increased lymphocyte recruitment, airway inflammation, and mucus production when compared with OVA-tolerized mice (Fig. 2C, 2D). Taken together, these data indicate that systemic administration of CS abrogates the development of respiratory tolerance. Because the degree of systemic sensitization, determined by serum IgE levels, and the T cell proliferation were affected, we suggest that systemic administration of CS suppresses protective immune responses on both cellular and humoral levels.

**FIGURE 2.** Increased airway inflammation and AHR in OVA-tolerized mice treated in i.p. administration of CS. A and B, A group of BALB/c mice (n = 4) was treated i.n. with 100 µg OVA (OVA Tolerance) or PBS (OVA Sensitized) on consecutive 3 d (days 1–3). Some of the mice from the OVA tolerance group were also treated i.p. with 500 µg Pred. (OVA + Pred. i.p.) or 100 µg Dexa. (OVA + Dexa. i.p.) each on days 0 and 2. On day 12, all the mice were immunized in i.p. with OVA (50 µg) in alum (2 mg). On day 22, mice were subsequently challenged with OVA (50 µg) i.n., or PBS (neg ctrl), on consecutive 3 d and assessed for AHR by measuring PenH (A) or Rl and Cdyn (B). Negative control mice were treated with PBS only. Data are the mean ± SEM, representative of four experiments (n = 4) with *p < 0.05, **p < 0.005. C, BAL fluid from the mice in A was analyzed 24 h after AHR measurement. Results are shown as the total number of cells in BAL fluid. *p < 0.05, **p < 0.005. D, Lung histopathology. Lung tissue of mice from A was stained with H&E (upper panel) and analyzed for cell infiltration. OVA-sensitized and CS-treated mice show numerous inflammatory cells surrounding the airways in the lumen, whereas the OVA-tolerized control mice show normal airway and the surrounding parenchyma (lower panel). Lung tissue from the same mice was stained with PAS and analyzed for the presence of mucus. The production of mucus was important both in OVA-sensitized and CS-treated mice compared with OVA-tolerized mice. Arrows indicate the production of mucus in the lumen (original magnifications ×200; inset ×600). Eos, eosinophils; Lym, lymphocytes; Mac, monocyte/macrophage; PMN, neutrophils; Total, total cell number.
The i.n. administration of CS does not affect the development of mucosal tolerance

To analyze whether the route of administration of CS plays a role in its effects on the respiratory tolerance induction, we treated mice i.n. with Pred. or Dexa. during the induction of tolerance, as described in Fig. 1A. Inhaled CS had no effect on total or OVA-specific IgE production. This was in concordance with systemically applied CS (Fig. 1B). However, in contrast to systemically applied CS, i.n. administration of Dexa. or Pred. during the respiratory tolerance induction did not abrogate the respiratory tolerance. Indeed, in mice i.n. treated with CS during the tolerance induction, the Ag-specific T cell proliferation was not restored (Fig. 1D), even by using higher doses of i.n. CS (500 μg Pred. or 125 μg Dexa.) similar to what was administered systemically. In addition, in i.n. CS-treated mice, the severity of AHR (Fig. 3A, 3B), the type and number of immune cells found in the BAL (Fig. 3C), but also the airway inflammation and mucus production (Fig. 3D) were similar to those observed for the OVA-tolerized mice.

Altogether, these data indicate that the route of administration of CS rather than the dose has an impact on the respiratory tolerance induction.

Systemic, but not i.n. administration of CS affects the T cell cytokine secretion

We have previously shown that OVA-induced cytokine secretion by T cells is greatly reduced in OVA-tolerized mice (10). We therefore examined whether systemic or i.n. administration of CS during the induction phase of tolerance could affect these cytokine secretions. As expected, OVA-induced cytokine secretion (IL-4, IL-10, and IFN-γ) by T cells from mice treated with inhaled CS was greatly reduced, but this effect was reversed by the systemic administration of both Dexa. and Pred. (Fig. 4A–C). Moreover, systemic administration of CS induced a strong Th2 cytokine secretion, as seen by the IL-4/IL-10 cytokine secretion ratios when compared with OVA-tolerized mice treated with inhaled CS (which secreted more IL-10) (Fig. 4D). These data indicate that...
systemic administration of CS abrogates the development of respiratory tolerance by blocking the T cell cytokine secretion inhibition and by inducing a shift in the IL-4/IL-10 cytokine secretion ratios. Systemic and i.n. administration of CS modulates Teff:Treg ratio in opposing direction

Our previous studies have shown that respiratory tolerance is highly effective in preventing the development of airway inflammation and hyperreactivity through the development of Ag-specific adaptive Treg that express high levels of Foxp3 (8, 9). Recent data showed that the determination of the Teff:Treg ratio is a better indicator of the balance between immune tolerance and activation than the evaluation of Treg number alone (15). We therefore investigated the effects of systemic and i.n. CS treatment on the balance between Teff and Treg during the respiratory tolerance induction. We adoptively transferred mice with DO11.10 T cells prior to the OVA tolerance induction in presence or absence of CS, as described in Fig. 5A. We then measured the percentage of Teff (CD4+CD25+Foxp3+) and Treg (CD4+CD25+Foxp3+) among the DO11.10 T cells in the spleen, lung, and LN and determined the corresponding Teff:Treg ratios. In mice treated with inhaled CS, Teff:Treg ratios were found to be slightly decreased both in the LN and the spleen compared with what was observed in OVA-tolerized mice not treated with CS. In contrast, in systemically treated mice, the Teff:Treg ratios were found to be increased in the LN (Fig. 5B). These data clearly demonstrate that systemic, but not i.n. administration of CS drives allergen-specific T cells toward the Teff rather than Treg.

Discussion

In this study, we showed that whereas systemic administration of CS prevents the protective effects of respiratory tolerance on the development of AHR, local administration of CS just scarcely affects this process. Mucosal tolerance is highly effective in preventing airway inflammation, and is thought to limit immune responses against the large quantities of innocuous Ags that enter the lungs suspended in inspired air (16, 17). Whereas CS can reduce acute inflammation in allergy (2), CS may at the same time hinder the development of mucosal tolerance, which is an immune response that downregulates Th2-driven allergic pulmonary inflammation (10). CS have been shown to be highly effective in the treatment of inflammatory diseases by reducing cytokine production and the function of critical effector cells. In asthma, CS inhibit acute allergic inflammation and improve airway hyperresponsiveness in both mice and humans (3), by limiting cytokine production in T cells and epithelial cells, and by impairing the recruitment and growth of eosinophils and other inflammatory cells (18). By all measures, CS, delivered either systemically or locally in the respiratory tract, is the pharmacological choice for both acute and chronic asthma (19, 20). In this report, we show that the route of application of CS significantly alters their effect. We found that systemic treatment with Dexa. or Pred. prevented the development of respiratory tolerance, resulting in Ag-specific proliferation and increased OVA-specific IgE production and Th2 cytokine production, allowing AHR to develop. CS might enhance Th2-allergic sensitization by several mechanisms. First, CS have been shown to potentiate IL-4-induced polyclonal IgE synthesis by peripheral blood B cells in vitro (21–

FIGURE 4. Effect of systemic versus local administration of CS on cytokine production. A group of BALB/c mice was treated, as described in Fig. 1A. Briefly, BALB/c mice were treated i.n. with OVA on days 0-2 (induction of tolerance). Positive controls received no i.n. OVA. Dexa. and Pred. group received three i.n. or two i.p. administrations of Dexa. (i.n., 25 μg; i.p., 100 μg) or Pred. (i.n., 125 μg; i.p., 500 μg) and were treated i.n. with OVA. On day 12, mice were immunized i.p. with OVA. Negative controls were treated with PBS alone. On day 22, mice were sacrificed and their splenocytes were restimulated in vitro (5 controls were treated with PBS alone. On day 22, mice were sacrificed and

FIGURE 5. Route of administration of CS influences the Teff:Treg ratio during the tolerance induction. A. Protocol of immunization and adoptive transfer of DO11.10 T cells. Briefly, 0.5 × 10^6 DO11.10 T cells were adoptively transferred into naïve BALB/c mice on day 0. The mice were then treated i.n. with 100 μg OVA on consecutive 3 d (days 1–3). Some of the mice from the OVA tolerance group were also treated i.p. with 100 μg Dexa. (OVA + Dexa. i.p.) on days 0 and 2 or i.n. with 25 μg Dexa. (OVA + Dexa. i.n.) on days 1–3. Four days after the last i.n. administration, the cells from cervical LN, spleen, and lung were isolated and stained for the extracellular markers CD3, CD25, CD4, and DO11.10, and intracellular maker Foxp3. B. Gated on the CD4+DO11.10+ T cells, we evaluated by flow cytometry the percentage of Teff (CD25+Foxp3-) and Treg (CD25+Foxp3+). Results are shown as the ratios of Teff:Treg in the LN, spleen, and lung with n = 3.
In vivo, CS treatment has been shown to increase serum IgE levels transiently (26, 27). In addition, CS therapy has been shown to potentiate Th2 differentiation, by either reducing IL-12 secretion from APCs (4, 5, 24, 28–33) or by directly suppressing Th1 cell polarization (34). CS do not appear to diminish the expression of costimulatory molecules on DCs (32), allowing CS-treated DCs to present Ag to T cells and enhance immune deviation toward Th2 differentiation. Furthermore, respiratory tolerance has been found to be dependent on IL-10–producing tolerogenic DCs (14), IL-10–producing T cells, and Foxp3+ Treg cells (8, 9). Previously, we found that systemic application of Dexe. blocks the development of T cell tolerance as a result of the elimination of IL-10–secreting tolerogenic DCs (10). In this study, we found that systemic administration of CS therapy might inhibit the development of respiratory tolerance by decreasing the allergen-specific Foxp3+ Treg development, but not the allergen-specific Teff development, resulting in a shift of the Teff:Treg balance toward the T cell activation. This shift of the Teff:Treg balance induced by the systemic administration of CS occurs in the second lymphoid organs where the immune cells responsible for the tolerance induction develop. Therefore, our results suggest that Foxp3+ Treg development is more sensitive to systemic CS therapy than Th2 cell development (35), resulting in enhanced Th2 cell, but reduced Foxp3+ Treg development following systemic CS treatment.

In contrast, we found that local administration of Pred. or Dexe. has no effect on respiratory tolerance induction. Inhaled CS were unable to inhibit T cell tolerance and had no effect on total or OVA-specific IgE production, cytokine secretion, and AHR development. However, we found that inhaled CS decrease the allergen-specific Teff:Treg ratios mainly through the increase of the Treg development. This correlates with one report describing an increased Treg activity in asthmatic patients treated with inhaled glucocorticoids, and significantly increased FOXP3 mRNA expression was found in unstimulated peripheral blood CD4+ T cells after steroid treatment (36).

It is well known that asthmatic patients treated with CS received a lower dose when treated locally than systematically to afford the same quantity of CS to reach the lungs. Therefore, to mimic human treatment, we treated mice with higher doses of CS when administered systematically than i.n. However, despite different doses of CS being used in function to the route of administration, this cannot explain the different effect of local versus systemic administration of CS on the respiratory tolerance induction. Indeed, we found that respiratory tolerance was also abrogated in mice treated systematically with low doses of CS as administered i.n. despite a decrease of the efficiency. In addition, treatment of mice i.n. with higher doses of CS, same as administered in i.p., did not abrogate the respiratory tolerance induction.

This suggests that the route of administration rather than the doses administered affects the respiratory tolerance induction. The difference between inhaled and systemically applied corticoids may consist of intrinsic activity as well as local and systemic metabolism. Particularly the pharmacokinetic and pharmacodynamic properties of inhaled CS could be of significant importance in this respect. Also, ~10–20% of a given dose from a metered dose aerosol inhaler reaches the airways, with the remaining 80–90% being deposited in the oropharynx and subsequently swallowed (37). Systemic bioavailability of inhaled drugs may thus arise from absorption from the gastrointestinal tract or the lung. In contrast, systemic treatment with CS may lead to significantly higher serum concentrations. Consequently, even if equivalent doses of CS are administered locally and systemically, when injected systemically, higher doses of CS can reach the second lymphoid organs and thus interfere with the development of the immune cells responsible for the tolerance induction. Furthermore, whereas application of inhaled CS inhibits Teff cells in the lung, the long-term induction of tolerance and subsequent physiologic infiltration of Tregs in the lung may not occur in the presence of systemic immunosuppression. Finally, our studies have very important implications regarding the route of administration of CS in the treatment of allergic inflammatory diseases in humans. Both systemic and topical administrations of CS have clear anti-inflammatory effects and are effective for controlling the symptoms of asthma and associated airway inflammation by limiting the function of Th2 effector cells and eosinophils in acute asthma (1–3). However, the inhibitory effects of systemic administration of CS on Treg development may impart insidious side effects that may inexcorably enhance the severity of subsequent immune responses that occur on re-exposure to allergen. Allergen-specific Treg are thought to be present in higher frequency in nonallergic than in allergic individuals, to be responsible for downmodulating allergic responses and asthma (16), and appear to be induced with allergen immunotherapy (38, 39). Therefore, inhibition of Treg development by CS would block these normal regulatory mechanisms that control allergy and asthma.

Our results show that inhaled CS do not drive the allergen-specific Teff:Treg ratio toward T cell activation. We therefore suggest that local administration of CS has no unwanted long-term effects and allows to limit the enhancement of the severity of subsequent immune responses that occur on re-exposure to allergen, maybe contrary to systemic administration of CS.

However, inhaled CS treatment is by no means curative, as the symptoms of asthma reappear when therapy is stopped. The development of pathogenetically aimed treatment strategies remains necessary.

Taken together, our findings from these experiments could shed some light into the highly contradictory field of CS effects in allergic diseases.

Acknowledgments
We thank Viola Kohlrautz and Christine Seib for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References


